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JELLYFISH

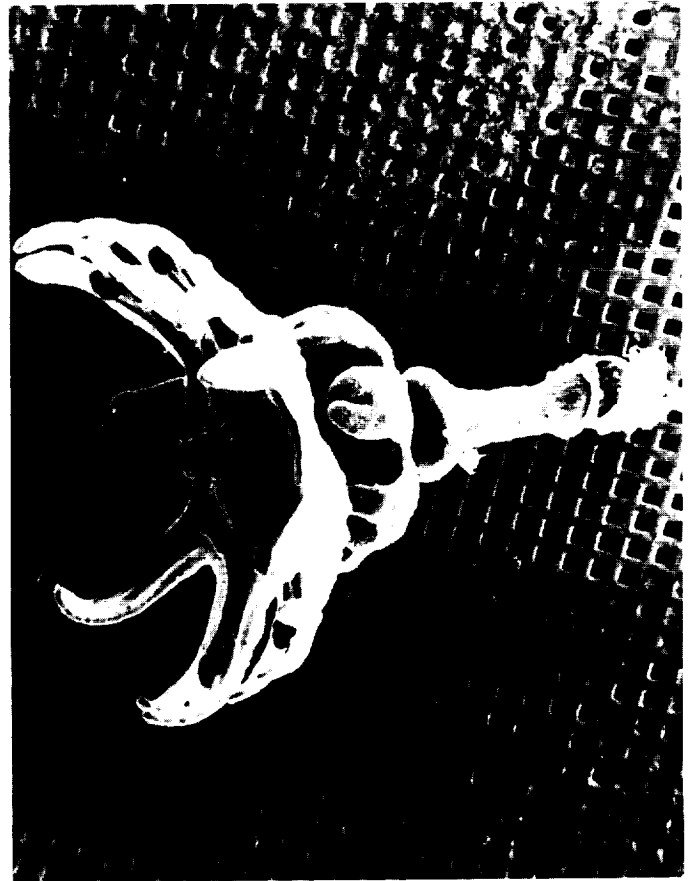
*Special Tools for Biological Research
on Earth and in Space*

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When I was a youngster beachcombing in Galveston, Texas, I often saw large numbers of jellyfish washed up along the beach. I viewed the jellyfish, then, as most people do today, as nuisances. They cluttered the sand, often obscuring the more interesting seashell specimens I was collecting, and, of course, in general, they fouled the beach. It was years later, while studying at the University of Texas, Austin, that I began to realize what extraordinary creatures the jellyfish are and what great tools for biological research they could be.

My serious studies of the jellyfish, *Aurelia aurita* (moon jellies or plates) began in 1962 using a strain collected at Corpus Christi, Texas. I learned that the small polyp form of the *Aurelia* can be reared easily in small dishes of sea water (or artificial sea water) while being fed newly hatched brine shrimp (*Artemia salina*). Given good care, which involves changing the polyps into clean dishes and sea water after each feeding (now being done in my laboratory by ODU student Suzanne Davis), the jellyfish polyps will live indefinitely while continuously reproducing through budding. Indeed, I still have some of the Texas strain of jellyfish collected so many years ago!

The most intriguing nature of the jellyfish polyps, to me, is their ability to metamorphose, giving rise to tiny immature medusae called ephyrae which have a different form or shape from the polyps. In order to study the process of metamorphosis in the jellyfish, we had to be able to induce animals to metamorphose on command. After many years of research, we achieved this purpose by discovering that the jellyfish needed iodine in order to make a thyroid-type hormone required for metamorphosis. Today, we can "make" as many ephyrae as we need for our research studies whenever we need them by administering iodine and controlling their environmental temperature. This ability to control metamorphosis of polyps with iodine treatment led to the development of an exciting new test system, the *Aurelia* Metamorphosis Test System.



A nearly mature ephyra is developing at the top of a metamorphosing polyp. Less mature ephyra is underneath it. This is magnified approximately 50X.

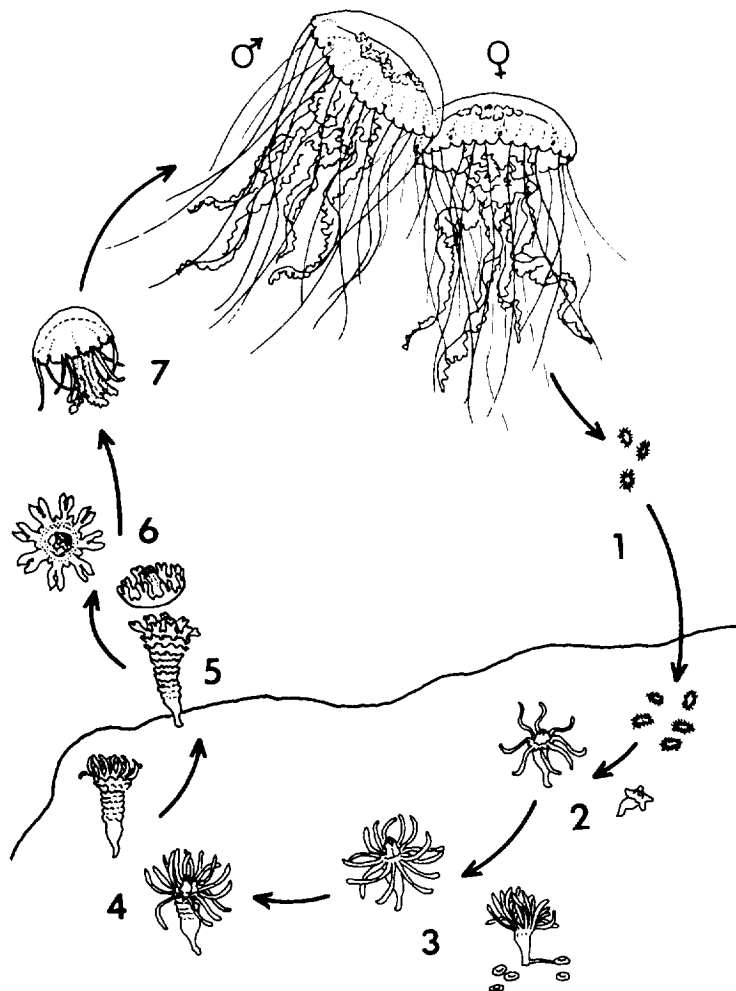
To date, we have used the *Aurelia* Metamorphosis Test System to determine the subtle effects of hydrocarbons found in oil spills (sponsored by the Department of Energy) and the effects of X-irradiation on developing ephyrae (sponsored by Eastern Virginia Medical School and done in collaboration with Mr. Mike Prokopchak). Currently, we are using this test system to determine the effects of the gravity-less environment of outer space on the development and behavior of ephyrae (sponsored by NASA). For this purpose, I am studying the effects of clinostat rotation on development of the ephyrae and their gravity receptors; we are looking at the behavior of the ephyrae during 0 gravity achieved for short intervals of 30 seconds in parabolic flight (in collaboration with Dr. Charles Oman, Massachusetts Institute of Technology); and we are planning exposure of developing ephyrae and of mature ephyrae to the gravity-less environment of outer space via a six or seven day shuttle experiment.

People ask me, "Why should we fly the jellyfish in outer space?" I reply that the jellyfish ephyrae form simple gravity receptor structures which resemble, in a less complicated way, the gravity receptor structures of higher organisms, including humans. The gravity-less environment of outer space is still a new, relatively unexplored frontier. We do not know, yet, how this gravity-less environment will affect the development of biological organisms or their gravity receptor structures. Indeed, we do not know what role gravity may be playing in our own development on earth. By comparing development of the ephyrae in space with that of ephyrae on earth, we expect to be able to answer questions about the importance of gravity which would be impossible to answer without the availability of the shuttle to take the jellyfish and other organisms into outer space.

It's a long journey for the jellyfish from the depths of the ocean to outer space, yet the jellyfish polyps and ephyrae are especially suited for the trip. They are tiny, require little or no care during their week-long journey, and have the capability of forming the special gravity-sensing structures. If the gravity-receptors do not form in outer space, we will deduce that gravity was needed for normal development (having controlled for other factors) and that gravity plays an important role in the normal development of these structures on earth. If gravity receptors do form in outer

space, we will study them in detail using various types of microscopes, including the electron microscope, to determine whether they developed normally in space as compared with controls on earth.

When I walked the beach of Galveston and avoided the jellyfish washed up there, I knew nothing about jellyfish metamorphosis or even that jellyfish made gravity-sensing structures. Indeed, the first space craft had not yet gone into outer space. Through basic science research of the jellyfish over the years, however, we learned about the special features of the jellyfish which make them especially valuable for gravity-related research. Today, when I walk the beaches of Norfolk and Virginia Beach, I think about the vast numbers of unexplored organisms in the ocean (and on the beaches) and I wonder about their special features and how they could be used to answer basic fundamental questions about living organisms. What a wonderful opportunity for scientists to have the use of such a smorgasboard of exotic marine animals with such a wealth of special features for exploration. Who knows what important questions will be answered through these aquatic organisms about life on earth and in space today and in the future!



Jellyfish life cycle, above, and drawing of comb jelly on page 4 are from Common Jellyfish and Comb Jellies of North Carolina by Frank J. Schwartz and illustrated by

Leslie Barling. Copies are available for 50¢ by writing P.O. Box 809, Moorhead City, NC 28557.

EFFECTS OF CLINOSTAT ROTATION ON AURELIA STATOLITH SYNTHESIS

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Aurelia ephyrae develop eight graviceptors (rhopalia) during their metamorphosis from polyps, which are used for positional orientation with respect to gravity.

In three experiments for each speed of 1/15, 1/8, 1/4, 1/2, 1, and 24 rpm, groups of six polyps were rotated in the horizontal or vertical plane (control) using clinostats. Other controls were kept stationary in the two planes. Ten ephyrae from each group were collected after 5-6 days at 27°C in iodine and the number of statoliths per rhopalium were counted. Statistical analyses of statolith numbers revealed that horizontal clinostat rotation at 1/4 and 1/2 rpm caused the formation of significantly fewer statoliths per rhopalium than were found in controls. The finding that these slow rates of rotation reduces statolith numbers suggests that the developing ephyrae were disoriented with respect to gravity at these speeds, causing fewer statocytes to differentiate or to mineralize.

INTRODUCTION

Using the *Aurelia* Metamorphosis Test System (Spangenberg, 1984), we have been investigating the effects of clinostat rotation in the horizontal plane on the development of ephyrae and the synthesis of their statoliths.

Aurelia polyps are especially suited for gravity-related research because they are very small (2-4 mm), form ephyrae with gravity sensing structures (rhopalia) in 5-6 days, and can be used for clinostat studies. During iodine-induced metamorphosis (Spangenberg, 1967), ephyrae develop in sequential order from the oral to the aboral end of the polyps. Eight rhopalia with sacs of statoliths at their distal ends form per ephyra. These statoliths are composed of calcium sulfate dihydrate (Spangenberg and Beck, 1968) and only one statolith forms per statocyte.

METHODS

Two clinostats, made according to the design of Tremor and Souza (1972), were used for these studies. Jellyfish polyps were impaled head

downwards on cactus spines embedded in paraffin in the conical bottoms of autoanalyzer capsules (Figure 1). The capsules were filled with 10^{-3} M iodine in artificial sea water (ASW) prepared according to Spangenberg (1967). The caps of the capsules were filled with paraffin to eliminate bubble formation in the capsules, and the cap-capsule junction was covered with pressure sensitive tape to prevent leakage of the solution or evaporation. The capsules were tightly held in a 9 inch long glass tube with plastic joiners and the tube was attached to the shaft of the clinostat.

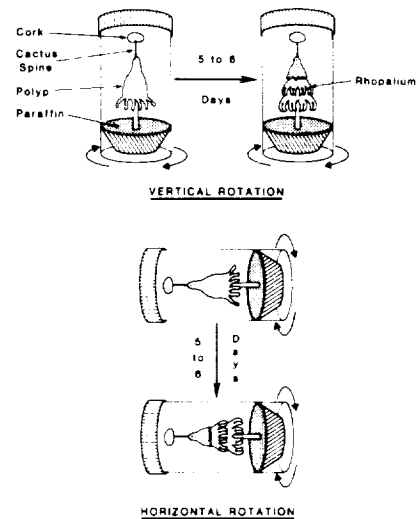


Figure 1. Orientation of the polyps and developing ephyrae during clinostat rotation.

Three tests were done for each clinostat speed using groups of 6 polyps (one per capsule) and speeds of 1/15, 1/8, 1/4, 1/2, 1, and 24 rpm were used. For each test, polyps were (1) rotated in a horizontal plane; or (2) rotated in the vertical plane; or (3) kept stationary in the horizontal position and placed near to (1); or kept stationary in the vertical position and placed near to (2). After 5-6 days at 27°C, the polyps formed ephyrae in all of the groups, and the ephyrae were removed from the spines in the capsules and squashed in a wet film. The excess ASW was removed to flatten the animals, causing the statoliths to spread, so that the number of statoliths per rhopalium per ephyra could be counted and recorded (Figure 2). Statistical analyses were done on the number of statoliths formed per rhopalium using an ANOVA and the Duncan's New Multiple Range Test.

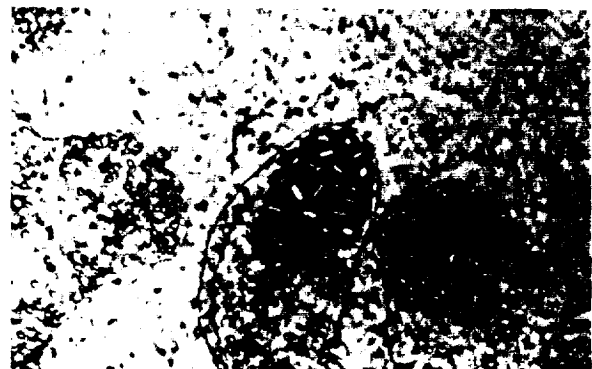


Figure 2. Statoliths spread in ephyra squash preparation.

RESULTS

Comparison of the numbers of statoliths formed by ephyrae which had developed during clinostat rotation in the horizontal plane with controls were made using an ANOVA which revealed significant differences between the groups at the $p < .01$ level and the Duncan's New Multiple Range Test. The latter test showed that those ephyrae which developed during rotation in the horizontal plane or 1/4 or 1/2 rpm had significantly fewer statoliths than controls which were rotated at these rates in the vertical plane or were kept stationary (Table 1). Ephyrae rotated in the horizontal plane at the other speeds of rotation did not have significantly different statolith numbers than controls.

Table 1. Numbers of statoliths in ephyrae which developed during clinostat rotation. (120 ephyrae in 3 tests per rotation speed).

Duncan's New Multiple Range Test				
Rotation Speed (rpm)	Clinostat (Horizontal Plane)	Clinostat (Vertical Plane)	Control (Horizontal Plane)	Control (Vertical Plane)
1/15	25.7	25.6	26.3	23.8
1/8	22.0	19.9	19.2	17.0
1/4	*20.2	25.1	25.0	27.3
1/2	*22.7	25.4	25.5	24.5
1	22.0	16.9	20.9	17.4
24	32.7	27.6	30.5	29.6

*Mean numbers of statoliths per rhopalium of ephyrae rotated in the horizontal plane which are significantly different from controls. $p = .01$

DISCUSSION

A variety of developmental and maturational effects have been found in plants and animals following clinostat rotation in the horizontal plane (Miquel, 1984; Brown, 1984; and Walgemuth and Grills, 1984). Clinostat effects have been found using both slow and fast clinostat rotation. The reduced statolith formation in the jellyfish ephyrae rotated at 1/4 and 1/2 rpm is a response to slow clinostat rotation. Other biological systems responding to slow clinostat rotation in the horizontal plane include: the formation of more developmental anomalies in *R. pipiens* and *X. laevis* following rotation of their egg at 1/4 rpm (Tremor and Souza, 1972); disturbed tobacco protoplast regeneration by rotation at 4 rpm (Iversen and Baggerud, 1984); and the delay of flower formation, seed production, and seed maturation which was found by Hoshizaka (1984) following rotation of *Arabidopsis* following clinostat rotation at 1/4 rpm.

A feature common to clinostat studies is the finding (as in the jellyfish statolith research) that rotation of organisms at some clinostat speeds elicit biological responses whereas no response occurs following rotation at other speeds. Lyon (1979) while referring to his experiments with *Coleus* and tomato plants reported that the difference in rotational time require-

ments for organs with different patterns of growth and hormonal controls illustrates the impossibility of setting a fixed rule for optimal rate of rotation of a clinostat. Iversen and Baggerud (1984) were unable to decide from their experiments which type of clinostat (fast or slow) provides the best simulated microgravity environment. These authors concluded that the response of their organisms indicated them to be a sensitive tool for studies of gravity effects which should be further tested in a real microgravity environment. Gruener (1984) also concluded that "it is impossible at present to assess the fidelity with which clinostat rotation simulates zero hypogravity encountered in space". Brown (1984) compared biological responses of plants rotated on the clinostat with those exposed to the microgravity environment of space. He found that circumnutation in plants occurred more vigorously in space than on the earth-based clinostat.

The mechanisms through which clinostat-rotation in the horizontal plane reduces statolith numbers in developing ephyrae is not known. In other organisms, such rotation has been reported to: cause mixing of intracellular constituents in frog eggs (Tremor and Souza, 1972); reduce movement of an auxin in *Coleus* and tomato plants (1970); significantly alter the functional interactions between the elements of a prototypic synapse in cultured cells of *X. laevis* (Gruener, 1984); cause a decrease in the content of starch grains in chloroplasts in protoplasts of tobacco using the slow but not the fast clinostat and to produce minor differences between protein patterns of rotated protoplasts and controls (Iversen and Baggerud, 1984).

Statolith synthesis in rhopalia of *Aurelia* ephyrae appears to be sensitive to disorientation of the organisms with respect to gravity. It is, therefore, possible that statolith synthesis will also be affected by exposure of developing ephyrae to the microgravity of space. Indeed, changes occurring in rhopalia following development in space could provide information which can lead to the identification of mechanisms through which microgravity affects statolith synthesis and through which gravity influences normal development of statoliths and rhopalia on earth.

ACKNOWLEDGMENTS

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REFERENCES

1. Brown, A.H. 1985. NASA Conf. Publ. 2336:37-39.
2. Gruener, R. 1985. NASA Conf. Publ. 2336:35-36.
3. Hoshizaka, T. 1985. NASA Conf. Publ. 2336:35-36.
4. Iversen, T. and C. Baggerud. 1984. The Physiologist. 27:S127-S130.
5. Lyon, C. 1970. Plant Physiol. 46:355-358.
6. Spangenberg, D. 1984. Mar. Environ. Res. 14:281-303.
7. Spangenberg, D. and C. Beck. 1968. Trans. Am. Neurosc. Soc. 87:329-335.
8. Spangenberg, D. 1967. J. Exp. Zool. 165:441-450.
9. Tremor, J. and K. Souza. 1972. Space Life Sci. 3:179-191.
10. Walgemuth, D. and G. Grills. 1984. The Physiologist 27:S99-S100.

Metabolism of Nonessential ^{15}N -Labeled Amino Acids and the Measurement of Human Whole-Body Protein Synthesis Rates¹

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ABSTRACT Eight ^{15}N -labeled nonessential amino acids plus $^{15}\text{NH}_4\text{Cl}$ were administered over a 10-h period to four healthy adult males using a primed-constant dosage regimen. The amount of ^{15}N excreted in the urine and the urinary ammonia, hippuric acid and plasma alanine ^{15}N enrichments were measured. There was a high degree of consistency across subjects in the ordering of the nine compounds based on the fraction of ^{15}N excreted (Kendall coefficient of concordance $W=0.83$, $P < 0.01$). Protein synthesis rates were calculated from the urinary ammonia plateau enrichment and the cumulative excretion of ^{15}N . Glycine was one of the few amino acids that gave similar values by both methods. *J. Nutr.* 116: 1651-1659, 1986.

INDEXING KEY WORDS ^{15}N • nonessential amino acids • protein synthesis

We are interested in the use of ^{15}N -labeled glycine as a tracer for determining the human whole-body protein synthesis rate. There are several variants, the principal ones being 1) methods based on the enrichment of the urinary ammonia (1,2), 2) decay curve analyses based on the decay of isotope enrichment in the urine or plasma following a single pulse, (3-5), 3) from the cumulative excretion ^{15}N excreted (6-8) and 4) a primed constant infusion-flux method (9).

Although several other methods involving ^{13}C -labeled amino acids have been described, the methods using ^{15}N are particularly suitable for use outside a hospital or clinical research center. The principal advantages of ^{15}N are: 1) ^{15}N can be used for single dosage-single sampling point methods, 2) serial sampling is not required and 3) there is no need to collect and analyze exhaled breath. Although other ^{15}N -labeled amino acids have

been used, for example, alanine (10), aspartate (11) and lysine (9, 12), glycine is the most frequently used ^{15}N -labeled amino acid because of its availability and relatively low price (13).

This study was designed to determine whether any of the other nonessential amino acids labeled with ^{15}N offer any advantages over glycine. We compared the glycine-based protein synthesis rates (PSR) using both the urinary ammonia plateau, ^{15}N enrichment and a cumulative excretion method against those found with other ^{15}N -labeled nonessential amino acids. We also compared the relative effectiveness of glycine in transferring ^{15}N to plasma alanine, urinary ammonia and urinary hippuric acid.

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1652

STEIN ET AL.

MATERIALS AND METHODS

The subject population consisted of four healthy adult males between 27 and 42 years of age. Informed consent was obtained in accordance with the policies of the Graduate Hospital and the University of Pennsylvania, Philadelphia, PA.

Eight ^{15}N -labeled nonessential L-amino acids plus ^{15}N ammonium chloride were tested. ^{15}N glycine (99%) and arginine (96%) were obtained from US Services/ICONS, Summit, NJ; ^{15}N alanine (99%); $^{15}\text{NH}_4\text{Cl}$ (99%) and ^{15}N glutamic acid (99%) from KOR Isotopes, Inc. (now ICN), Cambridge, MA; ^{15}N aspartic acid (98%), ^{15}N serine (99%) and ^{15}N amido-labeled asparagine (99%) from Merck and Co., St. Louis, MO. and ^{15}N amido-labeled glutamine (99%) from Cambridge Isotopes, Inc., Cambridge, MA. Chemical and isotopic purity were verified by gas chromatograph-mass spectrometry (GC-MS). The amino acids were converted to their *N*-acetyl isopropyl derivatives, and the spectra were compared with the appropriate spectra from unlabeled compounds.

The total dose of ^{15}N ranged between 20 and 100 mg ^{15}N per subject. For the more expensive amino acids, the first subject took between 35 and 70 mg ^{15}N . The results were then analyzed and a decision made as to the minimum amount needed to give accurate data, and that dose was given to subsequent subjects. The reason for doing so was to minimize the cost of the study. The total dose (20–100 mg ^{15}N) was divided into 13 equal aliquots and placed in gelatin capsules. Each capsule also contained 300 mg of sodium benzoate. Glycine is conjugated with benzoic acid in the liver to give hippuric acid, which is excreted in the urine. Thus the urinary hippuric acid provides a means of sampling the hepatic glycine pool (14).

The nine ^{15}N -labeled compounds were given in random order at approximately one per month to the four subjects. The study was started at 0700, after an overnight fast, with the subject voiding and taking a priming dose of four capsules. The remaining nine capsules were taken at hourly intervals thereafter. Each time the subject took a cap-

sule he drank 50 mL of Ensure (Ross Laboratories, Columbus, OH). Ensure is a defined formula diet containing 1 kcal/mL. The 50 kcal/h of Ensure was given because this caloric intake (500 kcal/10 h) approximated the subjects' normal caloric intake for the period 0700 to 1700. Subjects were permitted to consume noncaloric beverages (water, coffee or tea but with no cream or sugar) and nothing else from 0700 to 1700. At 1700, 7.0 mL of blood was drawn. Subjects collected a pooled urine for the period 0700 to 1230, after which time urine was collected approximately hourly until 1700. During the 10 h of the study the subjects performed their normal duties, which consisted of a combination of laboratory and office work.

Analytical methodology. Two methods were used for ^{15}N analysis. Plasma alanine enrichments were determined by GC-MS, and optical emission spectroscopy was used for the urinary nitrogen, ammonia and hippuric acid and the plasma urea. The sensitivity of the GC-MS and optical emission methods are ± 0.2 and ± 0.02 atom percent excess, respectively, in the range studied. Optical emission is the simpler technique, but requires a larger sample (3 μg of N_2) than the GC-MS method. Briefly, optical emission spectroscopy involves the irradiation of N_2 gas in a sealed 6-mm Vycor glass tube (Corning, Inc., Corning, NY) at 3 mmHg pressure with radio frequency energy. The N_2 molecules absorb the energy and reemit some of it as mauve light. The intensities of the emitted light at 297.68 nm ($^{14}\text{N}_2$) and 298.29 nm ($^{15}\text{N}^{14}\text{N}$) are compared by a standard monochromator-photomultiplier arrangement. This method requires that all samples be converted to N_2 gas, and this is usually accomplished by conversion to ammonia first and then oxidation to N_2 by alkaline hypobromite (15).

The blood urea nitrogen (BUN) was determined by the urease method using Sigma diagnostic kit #640 (Sigma Chemical Co., St. Louis, MO). For determination of the isotopic enrichment of the BUN, water (1 mL) and urease solution (2.0 mL, 0.21 mg urease/mL in 0.1 M phosphate buffer, pH 6.5) was added to plasma (2.0 mL). After incubating for 30 min at 37°C, K_2CO_3 (5 mL)

and 2-octanol (8 drops) were added. The ammonia was removed by aeration and collected in 0.1 N H₂SO₄ (1 mL). Total urinary nitrogen was measured on 1 mL of urine by the Kjeldahl method. Urinary ammonia was isolated from urine (5 mL) by adding saturated K₂CO₃ (5 mL) and aerating was done as described above for the BUN. The ¹⁵N enrichment of the urinary ammonia, total urinary nitrogen for each void (Kjeldahl distillates) and ammonia from the urease reaction on the BUN were determined by optical emission spectroscopy as previously described (15, 16).

Hippuric acid was isolated from the urine by crystallization after adjusting the urine concentration to 30% with sodium chloride (16). Occasionally the hippuric acid did not crystallize out. For those samples the urine (5 mL) was adjusted to pH 1 with concentrated H₂SO₄ and the urine extracted three times with ether (5 mL). The ether extracts were combined and evaporated to dryness. The pale straw residues from either the salt precipitation or the ether extractions were dissolved in minimal hot water. On cooling at 4°C, white needle-shaped crystals of hippuric acid separated out. The hippuric acid was then converted to (NH₄)₂SO₄ by Kjeldahl oxidation and analyzed for ¹⁵N by optical emission spectroscopy as described above.

The enrichment of the plasma alanine was determined by converting the plasma amino acids to their *N*-acetyl isopropyl esters as previously described (15). GC-MS analysis was done on a Hewlett-Packard 5992A GC-MS in the selective ion monitoring mode (SIM, ref. 15). The fragments at 186 amu (atomic mass units) and 187 amu were monitored. The parent peak is at 186 amu.

Methods of calculation. Fraction of ¹⁵N excreted. The amount of the administered dose excreted (**e*) was calculated by summing the ¹⁵N excreted in each urine sample collected over the 10-h period and the ¹⁵N remaining in the body urea pool at 10 h. The latter was calculated from the blood taken at the end of the experiment. The size of body urea pool (in grams) was estimated from the BUN (in milligrams/100 mL) using an equation derived by Hume and Weyers (17):

$$\text{urea pool} = \text{UDS} \times 10^3 \times \text{BUN}$$

where UDS is urea distribution space and $\text{UDS} = (0.195 \times \text{ht in centimeters}) + (0.297 \times \text{wt in kilograms}) - 14.013$. Thus ¹⁵N in urea pool = UDS (in liters) × BUN (in grams/liter) × BUN ¹⁵N (atom percent excess) × 0.01.

Whole-body protein synthesis rates. The whole-body protein synthesis rate (PSR) was calculated from the urinary ammonia ¹⁵N enrichment at plateau (18) and from the total amount of ¹⁵N excreted (6–8). From the urinary ammonia, PSR was calculated as follows:

$$Q = *d/A \text{ and } Q = E + S$$

where *Q* is nitrogen flux in grams N/hour; **d* is rate of ¹⁵N glycine administration in grams N/hour; *A* is ¹⁵N abundance in the urinary ammonia at plateau (APE × 0.01); *E* is rate of N excretion in grams N/hour; *S* is rate of protein synthesis in grams N/hour; and APE is atom percent excess ¹⁵N.

From the total ¹⁵N excreted (cumulative excretion), PSR was calculated as:

$$S = E_T (*d/*e - 1)$$

where **d* is amount of ¹⁵N given in grams N 10/hour; **e* is amount of ¹⁵N excreted (urine + BUN) in grams N 10/hour; *E_T* is amount of N excretion in grams N 10/hour; and *S* is rate of protein synthesis in grams N 10/hour.

The total amount of ¹⁵N excreted (**e*) was defined as described above. In this study, we assumed that there was no change in the BUN during the course of the study, and the amount of ¹⁵N in the tissue free amino acid pools at 10 h was negligible (5, 19).

RESULTS

A one-way, repeated-measures design analysis of variance was run on each measure: 1) % of ¹⁵N excreted, 2) PSR by a cumulative excretion method, 3) PSR from the urinary ammonia ¹⁵N plateau, 4) urinary NH₃ ¹⁵N enrichment at plateau, 5) plasma alanine ¹⁵N enrichment and 6) the urinary hippuric acid ¹⁵N enrichment. The results are summarized in figure 1 and table 1. The Kendall coefficient of concordance (*W*) was determined for each of the above measurements to provide a measure of the degree of consistency across subjects ("intersubject reliability") for the amino acids (table 1, ref.

1654

STEIN ET AL.

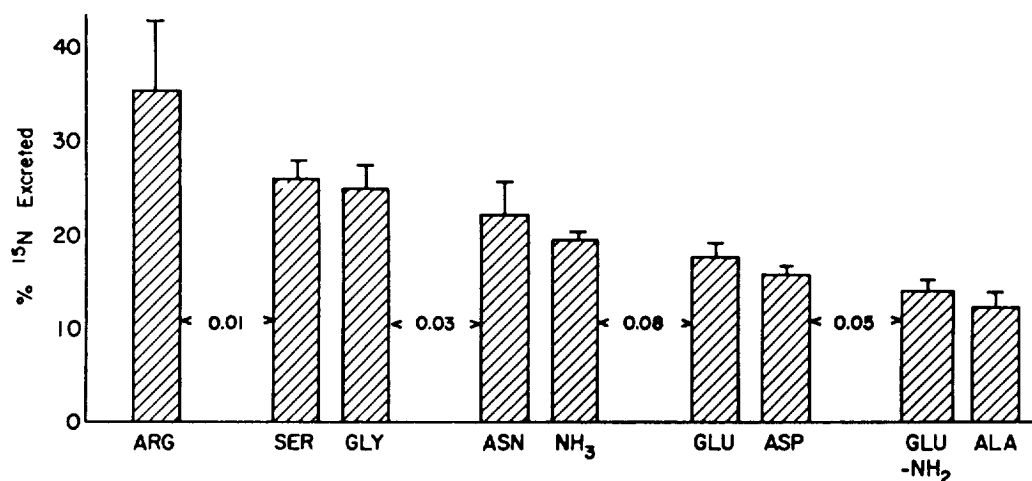


Fig. 1 Percentage of ¹⁵N excreted from various nonessential amino acids. Data is mean ± SEM, n=4. Numbers between groups are P-values.

20). The major findings of this study were:

1) There were significant differences in the fraction of ¹⁵N excreted among the various amino acids. The nonessential nitrogen compounds studied appeared to fall into five well-defined groups (figure 1).

2) There was high intersubject reliability in the ordering of the nine compounds based on the fraction of ¹⁵N excreted ($W=0.83$,

$P < 0.01$). All of the other ¹⁵N parameters except alanine also showed high intersubject consistency among subjects (table 1). As expected, parameters reflecting unlabeled nitrogen, such as nitrogen excretion and the BUN showed poor intersubject consistency ($W < 0.35$).

3) There was significantly less transference of ¹⁵N from guanido-labeled arginine

TABLE 1

Summary of synthesis rates calculated by flux (PSR_F) and cumulative excretion (PSR_T) methods and ¹⁵N enrichment of the urinary ammonia, hippuric acid and plasma alanine¹

	PSR _T	PSR _F	Urine hippuric acid ¹⁵ N	Urine ammonia ¹⁵ N	Plasma alanine ¹⁵ N
	g protein kg per d		APE		
Arginine	2.4 ± 0.7 ^a	27.6 ± 7.5 ^a	0.05 ± 0.02 ^a	0.06 ± 0.01 ^a	0.67 ± 0.15 ^{b,c}
Serine	4.4 ± 0.6 ^{a,b}	6.6 ± 4.5 ^{b,c}	2.03 ± 0.26 ^b	0.26 ± 0.06 ^b	0.71 ± 0.29 ^{a,b}
Glycine	4.3 ± 1.0 ^{a,b}	5.2 ± 1.9 ^{c,d}	1.93 ± 0.31 ^b	0.23 ± 0.06 ^b	1.71 ± 0.89 ^a
Asparagine	4.8 ± 2.1 ^{a,b}	9.2 ± 2.2 ^b	0.12 ± 0.01 ^f	0.16 ± 0.13 ^c	0.91 ± 0.69 ^{a,b}
NH ₄ Cl	7.2 ± 2.7 ^{b,c}	13.2 ± 2.5 ^a	0.26 ± 0.02 ^d	0.11 ± 0.02 ^c	0.58 ± 0.08 ^c
Glutamate	6.2 ± 1.2 ^b	7.2 ± 1.9 ^{c,d}	0.26 ± 0.04 ^{c,d}	0.19 ± 0.03 ^b	0.89 ± 0.10 ^{a,b}
Aspartate	6.9 ± 1.9 ^{b,c}	10.3 ± 4.5 ^{a,b}	0.23 ± 0.02 ^{d,e}	0.16 ± 0.05 ^c	0.83 ± 0.11 ^{a,b}
Glutamine	9.4 ± 2.7 ^c	5.1 ± 3.1 ^d	0.17 ± 0.02 ^e	0.25 ± 0.05 ^b	ND
Alanine	10.4 ± 3.3 ^c	9.2 ± 2.4 ^b	0.35 ± 0.02 ^e	0.15 ± 0.03 ^c	0.99 ± 0.42 ^a
Mean	6.1 ± 0.9	10.4 ± 2.6	0.60 ± 0.28	0.23 ± 0.05	1.07 ± 0.34
W (P)	0.79 (0.02)	0.72 (0.05)	0.97 (0.01)	0.84 (0.01)	0.42 (NS)

¹Values are means ± SEM. ND, no data. Unlike superscripts differ by $P < 0.05$. APE is atom percent excess (¹⁵N). W is the Kendall coefficient of concordance (20).

to ammonia or hippuric acid than from any other compound (table 1).

4) Two of the subjects excreted a very large proportion of the guanido N from arginine as urea (48 and 44 % vs. 23 and 21 %).

5) Less ¹⁵N from alanine and glutamine were excreted than from any of the other compounds tested (fig. 1).

6) Reasonable urinary ammonia ¹⁵N plateaus were obtained for the nine compounds (figure 2).

7) There was no close relationship between the protein synthesis rate based on the urinary ammonia ¹⁵N enrichment (PSR_F) and the value derived from the total amount of ¹⁵N excreted (PSR_T).

DISCUSSION

Selection of study conditions. The normal dietary intake between 0700 and 1700 of the four subjects ranged from breakfast (toast and coffee) and a meal for lunch to black coffee only. The dietary regimen (50 kcal/h) of Ensure was a compromise designed to approximate the mean normal nutritional intake of the four subjects for that period. During the study, the subjects performed their normal daily duties.

As a proportion of the oral diet given, the test amino acid may have represented up to a 50 % increase in the dietary amount of the test amino acid. Such relatively high dosages are routinely used in stable isotope tracer studies because of the relatively high enrichment levels needed for detection with most currently available analytical equipment (9). The "tracer" amino acid is mixed with the much larger pool of amino acids derived from protein breakdown so that the contribution to the amino acid flux approaches a tracer dose (~5 %; refs. 9, 18, 19, 21).

Excretion of ¹⁵N. There was high inter-subject reliability in the ordering of amino acids based on the fraction of ¹⁵N excreted ($W=0.83$, $P>0.01$). Furthermore, the compounds tested appeared to fall into five well-defined groups (fig. 1): 1) arginine, 2) serine and glycine, 3) asparagine and ammonia, 4) glutamate and aspartate and 5) glutamine and alanine (fig. 1). These groupings correspond to 1) an amino acid which is a very

close precursor of urea (arginine), 2) amino acids which are interconvertible and metabolized via ammonia and one carbon transfer reactions (serine and glycine), 3) compounds that are metabolized as ammonia (NH₄Cl and asparagine, which is hydrolyzed to ammonia in the gut), 4) amino acids whose carbon skeletons play key roles in intermediary metabolism (aspartate and glutamate) and 5) dual-function amino acids where the carbon skeleton is involved in intermediary metabolism and interorgan nitrogen transport (glutamine and alanine).

Although it was expected that a large proportion of the nitrogen from arginine would be metabolized to urea, the magnitude in two of our four subjects was surprising (48 and 44 % vs. 21 and 23 %). This may be due to human variability (22). Arginine has been reported to be a marginally essential amino acid (23).

Transference of ¹⁵N to other nonessential amino compounds. A high degree of consistency across subjects in the ordering of amino acids was found for the ¹⁵N enrichment of urinary ammonia and hippuric acid but not for alanine (table 1), possibly because of the lower sensitivity of the GC-MS analysis (± 0.2 atom percent excess). Like the alanine and hippuric acid ¹⁵N enrichment, the urinary enrichment of ammonia is another indicator of ¹⁵N transference but reflects both the enrichment of the free amino acid pools and the proportion of ammonia derived from the carrier amino acid. No other amino acid transferred significantly more ¹⁵N to urinary ammonia or plasma than glycine (table 1).

Calculation of protein synthesis rates. Although all estimates of the human body protein synthesis rate are based on the three-pool compartmental model developed by San Pietro and Rittenberg (5), there are many variants, each of which is based on slightly different sets of assumptions (18, 19, 24, 25). From all of these methods a consensus has emerged that the daytime human whole-body PSR is in the range of 1.5 to 6 g protein/kg per d. In this study we obtained values from the urinary ammonia plateau ¹⁵N enrichment and from total amount of ¹⁵N excreted. There was no apparent relationship between the whole-body PSR values derived

1656

STEIN ET AL.

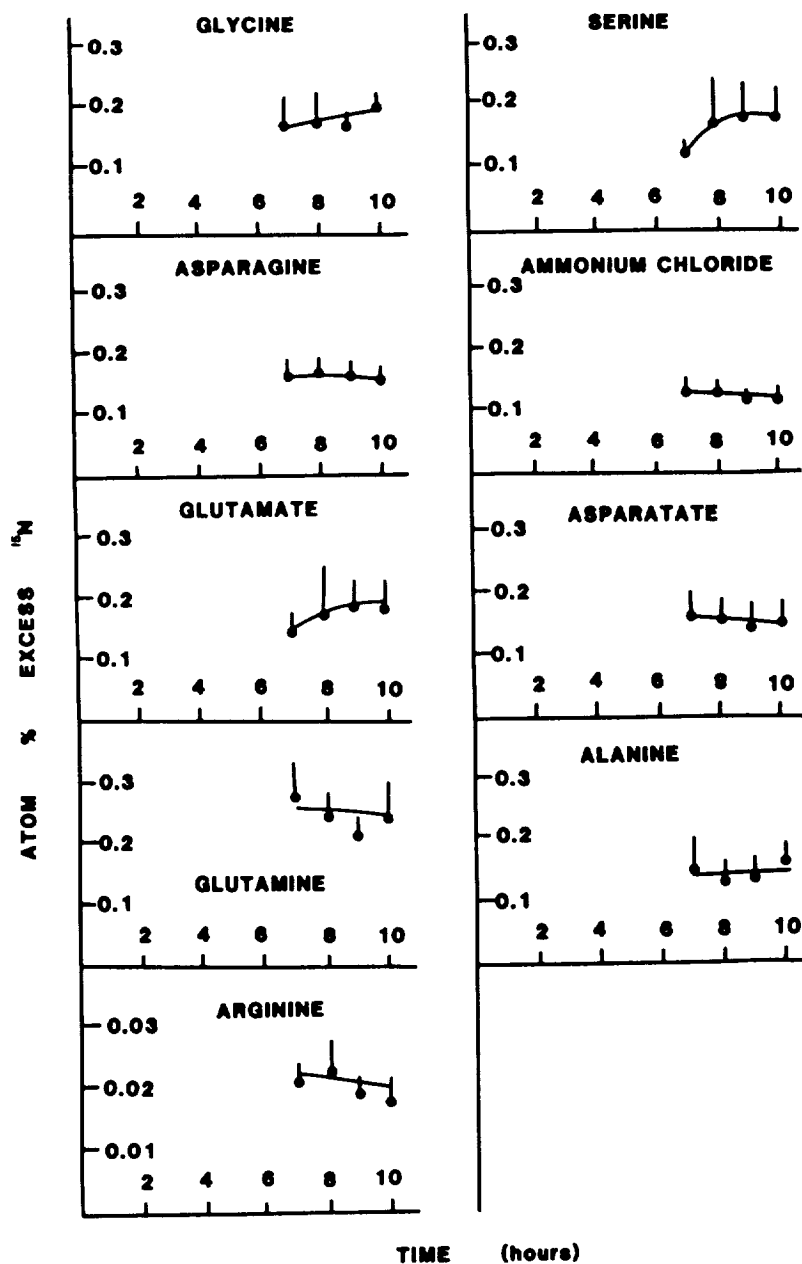


Fig. 2 Urinary ammonia enrichments for the urine specimens collected between 1230 and 1700. Data not normalized.

from the urinary ammonia and total excretion of ^{15}N .

Cumulative ^{15}N excretion. The basic assumption underlying the end point approach is that administered ^{15}N is parti-

tioned between end product (urea) and protein in the same proportion as total nitrogen, most of which is derived from endogenous protein breakdown. Although this issue has been addressed by several authors

previously, it is still not resolved for ^{15}N (7, 8, 13). The partitioning coefficient, or synthesis rates derived therefrom, should be independent of the route of isotope administration. Unless they are, one or both routes of isotope administration cannot reflect the composite endogenous nitrogen metabolism. Thus this study yields data on two criteria: 1) the calculated PSR should be in reasonable agreement with the value derived by other (carbon-labeled) methods, and 2) there should be reasonable agreement between the intravenous and orally derived values.

Most of the PSRs calculated by the cumulative excretion method in the present study are within the "consensus range" of 1.5 to 6 g protein/kg per d. The oral values found for glutamine and alanine (10.4 and 9.4 g protein/kg per d) in this study were high, although rates within the "consensus range" have been reported with the intravenous route (2.8 and 3.6 g protein/kg per day), respectively (10, 24). The oral aspartate and glutamate values are also elevated (table 1). These four amino acids are, as pointed out above, dual-function amino acids, and the second function can affect the partitioning of nitrogen. Previously, Tarunga (26) reported that essential amino acid nitrogen was unsuitable as a tracer because the nitrogen metabolism reflected the metabolism of the carbon skeleton. This study extends that conclusion to nonessential amino acids where the metabolism of the amino acid is dependent on both the partitioning between synthesis and excretion and the role in intermediary metabolism of the deaminated amino acid. Glycine meets the criteria of having no other major metabolic role if serine is regarded as part of the glycine pool. Glycine gives the same values orally or intravenously (25).

Urinary ammonia. There are at least two routes by which ^{15}N can be transferred to ammonia and then excreted in the urine. One is by entering, mixing and equilibrating with the body's free amino acid pools, a key assumption of both the flux and end product approaches. The other is by serving as a precursor to urinary ammonia without having attained isotopic equilibration in the tissue free amino acid pools. Different

amino acids contribute differently to the urinary ammonia, although the major precursor is plasma glutamine, and this may explain the lack of correlation between the methods (table 1, ref. 24). The values derived for the two methods were more similar for glycine than for any other ^{15}N compound tested.

The cumulative excretion method is much less dependent on the urinary ammonia enrichment because more than 90% of the ^{15}N is excreted as urea. These uncertainties, the lack of correlation with the more theoretically sound total-excretion-based values and the range and scatter in the flux-based values suggest that the cumulative excretion of total ^{15}N is the preferable approach.

Use of ^{15}N -labeled nonessential amino acids as tracers. There is some question as to how widely applicable single-isotope whole-body methods are. Anomalously high PSRs have been reported with glycine (8, 27), tyrosine (28) and leucine (29) in situations where there was hepatic insufficiency. The liver is particularly important in the mixing and equilibrating processes. Unexpectedly low (negative values) have been found with $[1-^{13}\text{C}]$ leucine in human subjects on low protein diets and during a simulated triathlon (30, 31). The fact that apparently anomalous results are found with several tracers suggests that the effect is not specific to the amino acid used but a failure of the model where liver insufficiency or other serious perturbations may result in compartmentation of the tissue free amino acid pools (27). The presence of any of these situations limits the applicability of single-isotope whole-body methods for the measurement of human whole-body protein synthesis rates.

The use of $[^{15}\text{N}]$ glycine. The PSR values derived from the two methods used in this study were closer for glycine than for any other amino acid and are similar to those found with ^{13}C -labeled amino acids. No other nonessential ^{15}N -labeled amino acid has been shown to be superior to glycine. $[^{15}\text{N}]$ glycine has several distinct advantages for studying human protein metabolism: 1) Methods involving $[^{15}\text{N}]$ glycine are technically the simplest (e.g., single-point assays (2)); 2) $[^{15}\text{N}]$ Glycine is relatively cheap and readily available in a pure state; 3) More is

1658

STEIN ET AL.

known about the metabolism of [^{15}N]glycine than any other ^{15}N -labeled amino acid; 4) Only [^{15}N]glycine offers the possibility of simultaneously measuring liver-originated plasma protein synthesis rates by using the urinary hippuric acid to determine the enrichment of the liver intracellular glycine pool (16).

LITERATURE CITED

- Garlick, P. J., Clugston, G. A. & Waterlow, J. C. (1980) Influence of low-energy diets on whole-body protein turnover in obese subjects. *Am. J. Physiol.* 238, E235-E244.
- Waterlow, J. C., Golden, M. H. N. & Garlick, P. J. (1978) Protein turnover in man measured with ^{15}N : comparison of end products and dose regimens. *Am. J. Physiol.* 235, E165-E174.
- Lapidot, A. & Nissim, I. (1980) Regulation of pool sizes and turnover rates of amino acids in humans: [^{15}N]glycine and [^{15}N]alanine single dose experiments using gas chromatography-mass spectrometry analysis. *Metabolism* 29, 230-239.
- Irving, C. S., Nissim, I. & Lapidot, A. (1978) The determination of amino acid pool sizes and turnover rates by gas chromatographic mass spectrometric analysis of stable isotope enrichment. *Biomed. Mass. Spectrom.* 5, 117-122.
- San Pietro, A. & Rittenberg, D. (1953) A study of the rate of protein synthesis in humans. II. Measurement of the metabolic pool and the rate of protein synthesis. *J. Biol. Chem.* 201, 457-473.
- Fern, E. B., Garlick, P. J. & McNurlan, M. A. (1981) The excretion of isotope in urea and ammonia for estimating protein turnover in man with [^{15}N]glycine. *Clin. Sci.* 61, 217-278.
- Fern, E. B., Garlick, P. J. & Waterlow, J. C. (1985) The concept of the single body metabolic pool of metabolic nitrogen in determining the rate of whole body protein turnover. *Hum. Nutr. Clin. Nutr.* 39C, 85-89.
- Stein, T. P., Buzby, G. P., Rosato, E. F. & Mullen, J. L. (1981) Effects of parenteral nutrition on protein synthesis in adult cancer patients. *Am. J. Clin. Nutr.* 74, 1484-1488.
- Wolfe, R. R. (1984) Tracers in Metabolic Research, Radioisotope and Stable Isotope/Mass Spectrometry Methods, chapters 12 and 13, pp. 151-170, Alan R. Liss Inc., New York.
- Birkhahn, R. H., Long, C. L., Fitkin, D., Jeevanadam, M. & Blakemore, W. S. (1981) Whole-body protein metabolism due to trauma in man as estimated by L-[^{15}N]alanine. *Am. J. Physiol.* 241, E64-E71.
- Crispell, K. R., Parson, W. & Hollifield, G. (1956) Protein synthesis rates as determined with [^{15}N]aspartic acid. *J. Clin. Invest.* 35, 154-165.
- Halliday, D. & McKeran, R. O. (1975) Measurement of muscle protein synthesis rate from serial muscle biopsies and total body protein turnover in man by continuous infusion of L-alpha-[^{15}N]lysine. *Clin. Sci.* 49, 581-590.
- Wutzke, K., Heine, W., Drescher, U., Richter, I. & Plath, C. (1983) ^{15}N -labelled yeast protein—a valid tracer for calculating whole body parameters in infants: a comparison between [^{15}N]yeast protein and [^{15}N]glycine. *Hum. Nutr. Clin. Nutr.* 37C, 317-327.
- Sperling, O., Wyngaarden, J. B. & Starmer, C. F. (1973) The kinetics of intramolecular distribution of ^{15}N -labeled glycine. A reappraisal of the significance of preferential labeling of N-(3+9) of uric acid in primary gout. *J. Clin. Invest.* 52, 2468-2485.
- Stein, T. P., Leskiw, M. J., Buzby, G. P., Giandomenico, A. L., Wallace, H. W. & Mullen, J. L. (1980) Measurement of protein synthesis rates with [^{15}N]glycine. *Am. J. Physiol.* 239, E294-E300.
- Stein, T. P., Leskiw, M. J. & Wallace, H. W. (1976) Measurement of half-life of human plasma fibrinogen. *Am. J. Physiol.* 234, E504-E510.
- Hume, R. & Weyers, D. (1971) Relationship between total body water and surface area in normal and obese subjects. *J. Clin. Pathol.* 24, 238-242.
- Golden, M. H. N. & Waterlow, J. C. (1977) Total protein synthesis in elderly people: a comparison of results with [^{15}N]glycine and [^{14}C]leucine. *Clin. Sci.* 53, 277-288.
- Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978) Protein Turnover in Mammalian Tissues and in the Whole Body, chapter 7, pp. 251-300, Elsevier/North-Holland, Amsterdam.
- Siegel, S. (1956) Nonparametric Statistics for the Behavioral Sciences, chapter 9, pp. 195-238, McGraw-Hill, New York.
- Mathews, D. E., Schwarz, H. P., Yang, R. D., Motil, K. J., Young, V. R. & Bier, D. M. (1982) Relationship of plasma leucine and alpha ketoisocaproate during a L-[^{1-13}C]leucine infusion in man. *Metabolism* 31, 1105-1112.
- Bessman, S. P. (1974) The justification theory for nonessential amino acids. *Nutr. Rev.* 34, 21-24.
- Barbul, A., Wasserkroug, H. L., Penberthy, L. T., Yoshimura, N., Seifter, E., & Levenson, S. (1984) Optimal levels of arginine in maintenance intravenous hyperalimentation. *J. Parenter. Enter. Nutr.* 8, 281-284.
- Golden, M. H. N., Jahoor, P. & Jackson, A. A. (1982) Glutamine production and its contribution to urinary ammonia in normal man. *Clin. Sci.* 62, 299-305.
- Stein, T. P. (1981) [^{15}N]glycine as a tracer to study protein metabolism in vivo. In: *Human Nitrogen Metabolism* (Waterlow, J. C. and Stephen, J. M. L., eds.), pp. 345-357, Applied Science Publishers, London.
- Tarunga, M., Jackson, A. A. & Golden, M. H. N. (1979) Comparison of ^{15}N -labelled glycine, aspartate, valine and leucine for measurement of whole body protein turnover. *Clin. Sci.* 57, 281-283.
- Stein, T. P., Ang, S. D., Schluter, M. D. & Nusbaum, M. (1983) Whole body protein turnover in metabolically stressed patients and patients with cancer as measured with [^{15}N]glycine. *Biochem. Med.* 30, 59-77.
- O'Keefe, S. J. D., Abraham, R. R., Davis, M. &

¹⁵N AND NONESSENTIAL AMINO ACIDS

1659

- Williams, R. (1981) Protein turnover in acute and chronic liver disease. *Acta Chir. Scand. Suppl.* 507, 91-101.
29. O'Keefe, S. J. D., Ramjee, G., Moldawer, L. L. & Blackburn, G. L. (1984) Parenteral nutrition in patients with liver failure, abstract #94, American Society for Parenteral and Enteral Nutrition 8th Clinical Conference, Las Vegas, NV.
30. Young, V. R., Robert, J. J., Motil, K. J., Mathews, D. E. & Bier, D. M. (1981) In: *Nitrogen Metabolism in Man*, (Waterlow, J. C. and Stephen, J. M. L., eds.), pp. 419-447, Applied Science Publishers, London.
31. Stein, T. P., Hoyt, R. W., O'Toole, M., Wolfe, R. R. & Hiller, W. D. B. (1985) Protein and Energy Metabolism during a Simulated Triathlon. 13th Int'l Congress of Nutrition, Brighton, UK, p. 170 (abs.).